

New functional tools for anti-thrombogenic activity assessment of live surface glycocalyx

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Materials and methods

Rat aorta harvest for functional assessment

All experimental animal work was approved by the Yale University Institutional Animal Care and Use Committee. Thoracic aortas were harvested from young adult (3 month-old) male Sprague Dawley rats. After induction of anesthesia with sodium pentobarbital, a transverse incision was made below the costal margin, entering the abdominal cavity. The heart and aorta were exposed after resecting the ribs. The portion of the aorta at the level of the renal arteries was partially incised to allow blood to be removed. The aorta was perfused through the right ventricle with 5 mL PBS, after which the thoracic aorta from the aortic valve to the diaphragm was excised and placed in Dulbecco's Modified Eagle Medium. The aorta was incised along its longitudinal axis to provide luminal surface exposure of the ECs and used without further treatment unless indicated otherwise.

HS surface digestion of rat aorta

Digestion of HS on the luminal EC surface was carried out *in vivo* in three rats. The renal portion of the aorta was incised to allow blood to be removed and the aorta was gently flushed with PBS. Heparinase digestion was restricted to the aorta by ligating the main aortic branches using Prolene 6.0 suture under magnification. Following the isolation of the aorta from its large branches, a 5 mL mixture of heparinase I, II and III (1000 mU each) was injected into the arch using a 28 gauge needle syringe. Following a 30 min incubation, the heparinase solution was aspirated and reserved for further LC-MS analysis. The aorta was excised for further analysis.

Culture of HUVEC monolayer and heparinase digestion

HUVECs were isolated from umbilical veins by treatment with collagenase. HUVECs were serially passaged 2 times in gelatin-coated tissue culture plastic using M199 medium supplemented with 20% FBS, 2% L-glutamine, 1% penicillin and streptomycin, 50 µg/mL endothelial cell growth supplement at 37 °C in a 5% CO₂ humidified air incubator. HUVECs were seeded onto 48 well plates until confluence (2-3 days). For heparinase digestion, cell monolayer was treated for 30 min with a heparinase I, II, and III mixture (1000 mU each) and the heparinase solution was aspirated and saved for further LC-MS analysis.

Functional analyses of aorta and cell culture monolayer's capacity to inactivate FXa and thrombin

Native vasculature and EC's ability to inhibit FXa and thrombin was determined per unit surface area (cm²) on native rat aorta (freshly harvested and untreated), heparinase-treated rat aortas (heparin was completely removed while preserving ECs), and HUVEC monolayer. Native rat aortas were cut to 1 cm² surface area and placed in a 48 well plates with the luminal EC surface facing upwards. Both the FXa and thrombin assays were limited to 16 min, thereby preserving cellular viability for the duration of the experiment.

The FXa assay measures a surface's ability to inactivate FXa, via the formation of the AT-FXa complex. Aortas or cells were incubated with 100 µL AT (25 mU/mL) in 50 mM Tris buffer (pH 8.4) for 5 min at 37°C. This allowed AT to bind surface-exposed HS and become activated. Thereafter, FXa (20 U/mL in 1 mg/mL BSA/PBS) was added and incubated for 2 min at 37°C, allowing activated AT to bind to FXa and inactivate FXa. The supernatants were then transferred into a 96-well plate where 25 µL of 1 mM chromogenic substrate S-2222 (Chromogenix) was added, mixed, and incubated for 10 min at 37°C and color change recorded every minute. Hence, the amount of FXa that was unbound to the cell or tissue surface is measured by the chromogenic reaction. Each sample group had three biological replicates, and each biological replicates was analyzed twice to provide technical duplicates.

The thrombin colorimetric assay measures a surface's ability to inactivate thrombin through the formation of AT-thrombin complex. Aortas or cells were incubated with 100 µL AT (25 mU/mL) in 50 mM Tris buffer (pH 8.4) for 5 min at 37°C. Thereafter thrombin (5 U/mL in PBS) was added and incubated for 2 min at 37°C. The supernatants were then transferred into a 96 well plate where 25 µL of 1 mM chromogenic substrate S-2238 was added, mixed, and incubated for 16 min at 37°C, and the color change recorded. Similar to the FXa assay, it is the amount of residual, non-complexed thrombin that is measured in this assay. Each sample group had three biological replicates, and each biological replicates was analyzed twice to provide technical duplicates.

Both FXa and thrombin assays used logarithmically increasing heparin (heparin sodium salt from porcine intestinal mucosa, MW 6000-30000 D, Sigma) dissolved in 100 µL 50 mM Tris buffer as controls. Briefly, 100 µL AT (25 mU/mL) in 50 mM Tris buffer (pH 8.4) was added to the heparin solution and incubated for 5 min at 37°C. Thereafter, FXa (20 U/mL in 1 mg/mL BSA/PBS) or thrombin (5 U/mL in PBS) was added and incubated for 2 min at 37°C, allowing activated AT to bind and inactivate FXa

or thrombin, respectively. The mixture was then transferred into a 96-well plate where 25 μ L of 1 mM chromogenic substrate S-2222 (for FXa assay) or S-2238 (for thrombin assay) was added, mixed, and incubated for 10 min at 37°C and color change recorded every minute.

Liquid chromatography mass spectrometry

The weight of HS, in both the aortic wall and in cell culture monolayer, was measured by liquid chromatography mass spectrometry (LC-MS) analysis. Briefly, as explained in the previous section a mixture of heparinase I, II, and III (a 5 mL mixture of heparinase I, II and III (1000 mU each) was added to either the aortas of three Sprague Dawley rats or three independent HUVEC monolayers grown in 48 well plates, and incubated for 30 min at 37°C. HS disaccharides were recovered by syringe aspiration, followed by centrifugal filtration on an YM-10 spin column, and lyophilizing the resulting retentate overnight. To ensure equal surface area of 1 cm², the length and diameter of perfused aortas were measured and the recovered retentate volume was normalized to 1 cm² surface area. The entire recovered retentate from HUVEC culture was used, as each 48 well represents 1 cm² surface area. Recovered unsaturated disaccharides were then labeled using 2-aminoacridine (AMAC) by adding 10 μ L of 0.1 M AMAC solution onto HS-derived disaccharides or to a mixture of the following 8 HS disaccharide standards: 0S, 2S, 6S, 2S6S, NS, NS2S, NS6S and TrisS (Iduron Ltd). Next, 10 μ L of 1 M NaBH₃CN was added in the reaction mixture and incubated at 45°C for 4 h. Finally, the AMAC-tagged disaccharides were diluted to different concentrations (0.5-100 ng) using 50% (v/v) aqueous DMSO. LC-MS analyses were performed on Agilent 1200 LC/MSD (Agilent Technologies) with a 6300 ion-trap and a binary pump and poroshell C18 column. Eluent A was 80 mM ammonium acetate and eluent B was methanol. The electrospray interface was negative ionization with a skimmer potential of -40.0V, a capillary exit of 40V, and a source temperature of 350°C.

Transmission electron microscopy (TEM)

The heparinase efficacy for removing glycocalyx was visually verified using TEM by comparing native and heparinase-digested aortas. Both native and heparinase-digested aortas were perfusion-fixed through the right ventricle by direct injection of 4% phosphate-buffered paraformaldehyde, pH 7.3, over 5 min. Aortas were then excised and immersed for 1 hour in 2.0% paraformaldehyde and 2.5% glutaraldehyde containing 0.075% ruthenium red, 75 mM lysine, and 0.1 M cacodylate pH 7.2 and then immersed overnight in the same solution. Following a rinse with 0.1 M cacodylate, tissues were post-fixed with 1% osmium tetroxide and dehydrated through a graded series of ethanol solutions, and embedded in LX112 Resin. All sections were viewed with a Zeiss EM-900, and imaged with a Mega View III Soft Imaging System.

Visualization of HS-specific proteoglycan

We developed a method for detection of HS chains directly through AT binding and validated it by direct visualization using immunofluorescent staining with a well-characterized HS-proteoglycan specific antibody. Fluorescein-labeled AT was prepared by mixing 1 mL of AT (25 UG/mg, purified from human plasma) with N-

hydroxysuccinimide-fluorescein (10 μ L, 830 nmol, Pierce) in 50 mM (pH 8.5) sodium borate. The reaction was mixed, incubated for 1 h, and dialyzed against distilled water. Heparinase-treated and untreated control rat aortas and cell monolayer cultures were fixed in 4% paraformaldehyde for 2 h, washed with PBS, and blocked in 1% FBS for 30 min. The samples were then incubated with fluorescein-AT (1:100) or anti-HS antibody (1:100, HepSS-1, Amsbio) for 1 h, washed with PBS, and incubated with Alexa Fluor 488 secondary antibody (Invitrogen) for 1 h. For nuclear staining, the samples were incubated with DAPI (Invitrogen) for 5 min, mounted with Vectashield medium (VectorLabs) and imaged using a Leica microscope.

Statistics

Each sample group had three biological replicates, and each biological replicates was analyzed twice to provide technical duplicates. Data were expressed as mean \pm standard error of means (SEM). Unpaired, two-tailed Student's t-tests were performed to evaluate whether the different groups were significantly different from each other. A value of $p \leq 0.05$ was considered statistically significant.